ISSN (print) 0093-4666 © 2014. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/129.25

Volume 129(1), pp.25–31 *July–September 2014*

Cladosporium species from hypersaline environments as endophytes in leaves of *Cocos nucifera* and *Vitis labrusca*

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Abstract — *Cladosporium dominicanum* and *C. halotolerans* were isolated in Pernambuco, Brazil, from healthy leaves of *Cocos nucifera* and *Vitis labrusca,* respectively. This is the first report of these species as endophytes and the first record of *C. dominicanum* for Brazil.

Key words —ITS, phylogenetic analysis, tropical plants

Introduction

Cladosporium was originally described by Link in 1816, and *Cladosporium herbarum* was selected from amongst Link's original species by Clements & Shear (1931) as the generic type (see also Bensch et al. 2010). With over 772 taxa, *Cladosporium* is one of the largest hyphomycete genera (Dugan et al. 2004). Bensch et al. (2012), who analyzed the genus phylogenetically, created an identification key and described 169 species. *Cladosporium* species are commonly found as plant and human pathogens and are also decomposers of food, paints, textiles, and organic matter (Ellis, 1971, 1976, Kwon et al. 2001; Liu et al. 2001). They occur as endophytes in plants of tropical regions (Costa et al. 2012) and are the most frequent endophytic fungi in some plants (Bezerra et al. 2012).

Cladosporium halotolerans is saprobic on various substrates and has been isolated in hypersaline water from subtropical regions, indoor environments, arctic ice, human and animal lesions, plants, rock, window frames, and mycorrhizal roots (Bensch et al. 2012). *Cladosporium dominicanum* was first isolated from hypersaline water and was also found as a saprobe on the surface of fruits (Bensch et al. 2012).

Here we report the first records of *C. dominicanum* as an endophyte in leaves of *Cocos nucifera* (and its first occurrence from Brazil) and of *C. halotolerans* as an endophyte in the leaves of *Vitis labrusca*.

Materials & methods

During February 2010 (dry season), healthy mature leaves of *Vitis labrusca* were collected from forest areas of the municipalities of São Vicente Férrer, Pernambuco, and in May 2012 (dry season), healthy mature leaves of *Cocos nucifera* were collected from forest areas of the municipalities of Goiana, Pernambuco.

Sterilization, isolation, and identification

In the laboratory, each leaf was washed gently in running water and soap. Leaf discs were cut with a sterile metallic cork punch (6 mm diam.), decontaminated with 70% alcohol for 30 sec and sodium hypochlorite solution (NaOCl) at 2% for 2.5 min, and twice washed with sterilized distilled water in order to remove the hypochlorite excess (Petrini 1996; modified technique). Six surface sterilized discs were transferred in triplicate to each Petri dish containing malt extract agar (MEA) + chloramphenicol (50 $mg.L^{-1}$) to prevent bacterial growth. The plates were incubated at room temperature (28 ± 2°C) and observed daily during 15 days for colony development. For asepsis control, 50 µL of water, used to remove hypochlorite, was plated in MEA to confirm surface disinfection (Pereira et al. 1993). Species identification was based on macro- and microstructural characteristics of the colony, according to Bensch et al. (2012). For each species (*Cladosporium halotolerans* and *C. dominicanum*), one isolate was deposited in the URM Culture Collection of the Universidade Federal de Pernambuco.

Molecular analyses

The fungi biomass was obtained from cultures grown on malt agar contained in test tubes and kept at 28°C for up to six days. All mycelium was removed from the test tube with the aid of a platinum loop; the material was transferred to 2 ml micro-tubes with screw caps to which were added 0.5 g of glass beads with two different diameters in the 1:1 ratio (acid-washed, 150–212 µm and 425–600 µm; Sigma, U.S. sieve). The material was crushed by stirring at high speed in a FastPrep.

Genomic DNA was extracted according to Góes-Neto et al. (2005). The material was washed with chloroform : isoamyl alcohol (24:1) and following homogenization of the material in CTAB buffer at 2%, besides isopropanol precipitation, washing in 70% ethanol, and re-suspended in 50 µL of ultrapure water.

Primers ITS1 and ITS4 (White et al. 1990) were used to amplify the ITS region. PCR reactions were carried out in 50 µL volumes containing 75 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 200 µM each dNTPs, 1 µM of each primer, and 2 units of Taq DNA polymerase (Fermentas, Maryland, USA); cycling parameters were 5 min at 95°C (1 cycle), 45s at 94°C, 1 min at 60°C, 1 min at 72°C (39 cycles), and a final elongation of 7 min at 72°C.

The final amplicons were purified with the PureLink PCR Purification Kit (Invitrogen). Sequencing was provided by the Human Genome Research Center (São Paulo, Brazil). Sequence data were compared to gene libraries (EMBL and GenBank) using BLASTn. The new sequences deriving from the species were deposited in the NCBI database under the accession numbers KJ000286 and KJ000287.

Phylogenetic analyses

The phylogeny was reconstructed by sequences of the ITS1+5.8s+ITS2 rDNA gene. The fungal sequences were aligned in ClustalX (Larkin et al. 2007) and edited with the BioEdit program (Hall 1999). Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al. 2004). Bayesian analysis (two runs over 1×10^6 generations with a burnin value of 2500) were performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), and maximum likelihood analysis (1000 bootstrap) was performed in PhyML (Guindon & Gascuel 2003), launched from Topali 2.5, using the GTR + I + G model. Neighbor-joining (established with the models cited above) and maximum parsimony analyses were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications.

The phylogenetic tree described in Zalar et al. (2007) was used as reference.

Results

Taxonomy

Cladosporium halotolerans Zalar, de Hoog & Gunde-Cim., Stud. Mycol. 58: 172. 2007.

Potato Dextrose Agar: Colonies after 14 days of culture at 25°C, presenting a growth of 53 mm, dark grayish green, reverse dark green. Conidiophores erect, lateral or terminal, olive to brown, septate, smooth, branched, 10–77.5 × 2.5–5 µm. Conidia in tandem, smooth, olive to brown, non-septate, globose, subglobose, or shortly limoniform (majority), slightly rough, 1.25–3.75 × 2.5–3.75 µm. Conidiogenous cells undifferentiated. Branch-primary conidia rare, with ≤2 distal scars; branch-secondary conidia, 0–1 septum (no majority), with ≤3 distal scars, 7.5–27.5 × 2.5–3.75 µm.

Malt Agar: Colonies after 14 days of culture at 25°C, presenting a growth of 59 mm, dark green grayish yellowish to dark green, reverse dark green. Conidiophores erect, lateral or terminal, olive to brown, septate, smooth, branched, 13–190 × 2.5–6.25 µm. Conidia in tandem, smooth, olive to brown, non-septate, globose to subglobose, 2.5–5 × 1.25–2.5 µm. Conidiogenous cells undifferentiated. Branch-primary conidia (2.5 × 2.5–3 µm) with up to 2 distal scars; branch-secondary conidia, 0–1 septum (no majority), with ≤2–3 distal scars, 3–22.5 × 2.5–3.75 µm.

Malt + 5% NaCl: Colonies after 14 days of culture at 25°C, presenting a growth of 50 mm, dark grayish green, reverse dark green, and with little sporulation. Conidiophores erect, lateral or terminal, olive to brown, septate, smooth, branched, 15–212.5 × 2.5 µm. Conidia in tandem, smooth, olive to brown, non-septate, globose (majority) to limoniform, rough 2.5–5 × 1.25–3.75 µm. Conidiogenous cells undifferentiated. Branch-primary conidia present; branch-secondary conidia, 0–1 septum, with up to 3 (mostly 2) distal scars, 10–25 × 3.75 µm.

Specimen examined: **BRAZIL**. **Pernambuco**: São Vicente Férrer, in healthy mature leaves of *Vitis labrusca* L. cv. Isabel, Feb 2010, T.E.F. Lima (URM6963; GenBank KJ000286).

Notes: *Cladosporium halotolerans* is known from Africa, Arctic, Asia, Australasia, Europe, North America, and Central and South America. This is the first recorded occurrence of the species as an endophyte.

Cladosporium dominicanum Zalar, de Hoog & Gunde-Cim., Stud. Mycol. 58: 169. 2007.

Potato Dextrose Agar: Colonies after 14 days of culture at 25°C, presenting a growth of 31 mm, green olive, velvety, reverse dark gray. Conidiophores erect, lateral or terminal, olive to brown, slightly verrucose, septate, branched or unbranched, 36–245 × 2–2.5 µm. Conidiogenous cells undifferentiated. Conidia in tandem, slightly verrucose, light brown, not septate, ovoid, 3.7–5.58 × 2.7–3.2 µm. Ramoconidia rarely formed; secondary cylindrical ramoconidia, 0–1 septate 14–28 × 2.5–3 µm.

Malt agar: Colonies after 14 days of culture at 25°C, presenting a growth of 34 mm, dark green, velvety, grooved, reverse dark gray. Conidiophores erect, lateral or terminal, olive to brown, slightly verrucose, septate, branched or unbranched, 50–160 × 2–2.5 µm. Conidiogenous cells undifferentiated. Conidia in tandem, slightly verrucose, light brown, non-septate, ovoid, 3.7–5.58 × 2.7–3.2 µm. Ramoconidia rarely formed; secondary cylindrical ramoconidia, 0–1 septate, 12–19 × 2.5–3 µm.

Malt + 5% NaCl: Colonies after 14 days of culture at 25°C, presenting a growth of 40 mm, light green, grooved, reverse dark gray. Conidiophores erect, lateral or terminal, olive to brown, slightly verrucose, septate, branched or unbranched, 45–107.5 × 2–2.5 µm. Conidiogenous cells undifferentiated. Conidia in tandem, slightly verrucose, light brown, non-septate, ovoid, 3.7–4.8 × 2.7–3.2 µm. Ramoconidia rarely formed, secondary cylindrical ramoconidia, 0–1 septate, 10–22.5 × 2.5–3 µm.

Specimen examined: **BRAZIL**. **Pernambuco**: Goiana, in healthy mature leaves of *Cocos nucifera* L., May 2012, R.J.V. Oliveira (URM6962; GenBank KJ000287).

Notes: *Cladosporium dominicanum* was previously known from Asia (Iran) and Central America (Dominican Republic). This is the first recorded occurrence of the species for South America (Brazil) and as an endophyte.

Molecular & phylogenetic analysis

The sequences generated from our fungal isolates grouped firmly in *Cladosporium*. Our isolate URM6962 (from leaves of *Cocos nucifera*) formed a clade with sequences from *C. dominicanum*, and isolate URM6963 (from leaves of *Vitis labrusca*) formed a clade with sequences from *C. halotolerans* (Fig. 1).

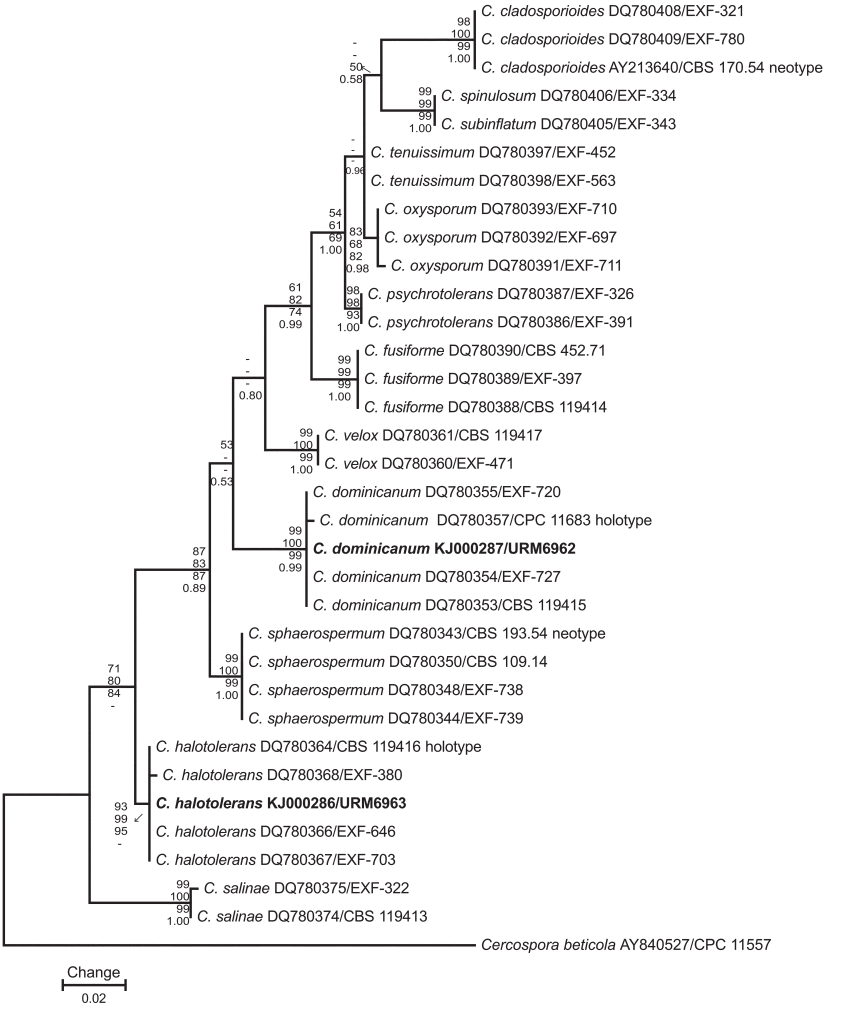

Fig. 1. Phylogenetic reconstruction of the sphaerospermum complex in *Cladosporium* obtained from sequences of the ITS region. Sequences are labeled with their GenBank accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. Only bootstrap values of at least 50% are shown.

In their review of *Cladosporium,* Bensch et al. (2012) showed that *C. halotolerans* and *C. dominicanum* belong to the *C. sphaerospermum* complex and are grouped close together.

In the Blastn analysis, our *C. dominicanum* sequence (KJ000287) showed 100% identity with sequence DQ780353 of the *C. dominicanum* ex-holotype culture (EXF752 = CBS 119415) and with two sequences (KC763352, KC845931) from Chinese isolates labelled "*C. sphaerospermum*". However, as sequence DQ780343 of the *C. sphaerospermum* neotype (CBS 193.54) showed only 97% identity with the sequence of our isolate URM6962, we conclude that sequences KC763352 and KC845931 in fact represent *C. dominicanum*.

In the Blastn analysis, our *C. halotolerans* sequence (KJ000286) showed 100% identity with sequence DQ780364 of the *C. halotolerans* ex-holotype culture (EXF572 = CBS 119416) and with 24 databank sequences labelled either "*C. cladosporioides*" (EF577236, AY361968, AB456576) or "*C. sphaerospermum*" (AB572909, AB572908, AB572903, AB572897, JN084018, AY625063, AM182174, AM182171, AM182168, AM176749, AM176685, EU759978, EU823317, JX156365, KC009836, JX839460, HQ263345, HQ248189, GU017501, JN253512, JN253512). However as sequence HM148003 of the *C. cladosporioides* neotype (CBS 17054) showed only 98% identity and the *C. sphaerospermum* neotype sequence showed only 96% identity with the sequence of our isolate URM6963, we conclude that the 24 database sequences probably represent *C. halotolerans* and not *C. cladosporioides* or *C. sphaerospermum*.

Acknowledgments

The authors thank Dr. José Luiz Bezerra and Dr. João Lúcio Azevedo for presubmission critical review. Special acknowledgements to Dr. Shaun Pennycook and Dr. Lorelei Norvell for their additional important corrections and suggestions to the manuscript. The authors also acknowledge the financial support provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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